

ON THE CHEMISTRY OF BACILLUS COLI
COMMUNIS.

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(FROM THE HYGIENIC LABORATORY OF THE UNIVERSITY OF MICHIGAN,
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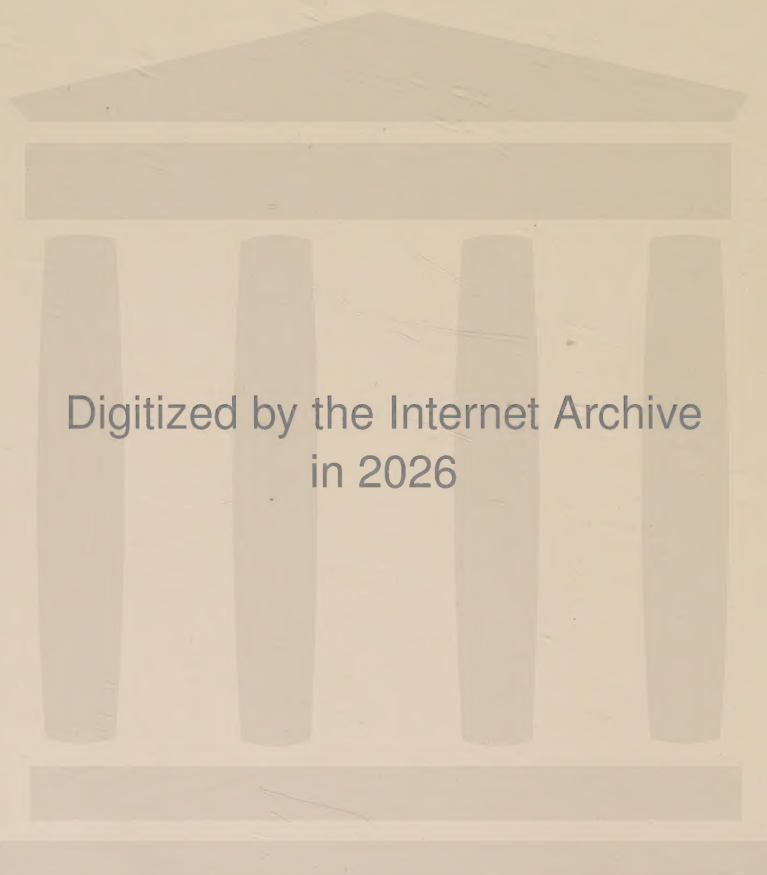
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ON THE CHEMISTRY OF BACILLUS COLI COMMUNIS.

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For the past few years, large incubating tanks have been in use in this laboratory for raising pure cultures of bacteria upon solid media. Each tank provides twenty square feet of growing surface, and yields under favorable conditions from thirty to fifty grams of air-dried cell substance. Thus it is possible to obtain sufficient material to make a systematic study of the chemical composition of the bacterial cell. It is proposed in this paper to give a review of the literature upon this subject, a *résumé* of some preliminary work upon the cell substance of *Bacillus coli communis*, a more detailed account of a toxic product split off by very dilute acid, and also of the preparation and purification of lysin from the same germ.

LITERATURE.

In the absence of data in regard to the colon bacillus, a study was made of the literature upon the chemical composition of other forms of bacteria, although it is not safe to assume that whatever has been ascertained concerning one species is true of another. Indeed, Nencki thinks there are greater differences in the chemical composition of nearly related forms among bacteria than among other orders of either plants or animals.

A large amount of work is reported upon the extracellular activity or the changes produced in the culture medium; indeed some of this study antedates the discovery of bacteria. Again, the application of the methods of physical chemistry to the various problems of bacterial chemistry opens a most profitable field of investigation. But both of these topics are beyond the scope of this review, except in such cases as have bearing upon the chemistry of the cell or of intracellular products. However,

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it is often very difficult to tell from the data given whether the products studied are intracellular or extracellular. This is especially true of work upon bacterial poisons.

ANALYSES. A number of the earlier observers have grown bacteria on solid media, removed the growth, and determined the moisture and ash, also the amount of substance soluble in alcohol and ether. The results are given in Table I; Table II includes a number of elementary analyses.

TABLE I.
MOISTURE, ASH, AND SUBSTANCE SOLUBLE IN ALCOHOL AND ETHER.

Germ.	Observer.	Medium.	H ₂ O. per cent.	Ash in dry subs. per cent.	Ash in moist subs. per cent.	Extract. per cent.
Putrefying bac.	Nencki	Gelatin	83.42	4.72*		6.04
Pneumo. bac.	Brieger	"	84.20	30.24		1.74
B. anthracis	Dyrmont	"	85.44			
B. prodigiosus	Kappes	Agar	85.45	13.47		4.83
B. xerosis	"	"	84.93	9.52		8.06
B. tuberculosis	Hammerschlag	Gly. agar	85.90	8.		3.84
B. prodigiosus	Cramer	Potato at 33°	75.83	9.31	2.26	
Do.	"	" 15°	80.02	12.52	2.63	
Do. 4 days old	"	"	79.56	11.33	2.41	
Do. 14 days old	"	"	82.55	13.77	2.38	

* Per cent. of dry substance after extraction with alcohol.

Kresling found that *Bacillus tuberculosis*, air-dried, lost 3.03 per cent. by drying at 100° to 110° C, or 3.018 per cent. by drying three months in a desiccator over sulfuric acid.

TABLE II.
ELEMENTARY ANALYSES.

Germ.	Observer.	Medium.	Carbon.	Hydro- gen.	Nitro- gen.	Sul- fur.	Phos- phorus.
B. tuberculosis	Hammerschlag	Gly. agar	51.62	8.07	9.09		
"	Levene	Beef broth	55.58	8.46	9.39	1.39	0.50
"	"	Mannite syn.	47.41	7.05	7.91	0.25	2.67
"	DeSchweinitz, Dorset	Beef broth	60.12	9.15	7.27		0.83
"	"	"	62.98	7.39	8.04		0.87
Putrefying bac.	"	Artificial	62.16	9.19	8.94	0.22	0.66
B. diphtheriæ	Nencki	"	53.82†	7.76	14.02		
B. anthracis	Dzierzgowski	Peptone	48.87‡	8.61	11.17	1.38	0.64
spores	Dyrmont	Gelatin					
threads	"	"	52.1	6.82	16.2		
Water bac. No. 28	Nishimura	Potato	50.98	6.75	11.05	1.02	
Erythema nodosum	Bovet	"	48.13	7.01	11.60		
B. Mallei	DeSchweinitz, Dorset	Beef broth	41.81	5.89	14.05	0.99	1.10
Swine plague	"	Peptone Do.	44.57	7.20	11.81		

* Calculated per cent. of dry substance.

† Free from ash and fat.

‡ After extraction with alcohol and ether.

On comparing these figures, one notices not only the diversity between different species, but the variations in different cultures of the same germ. Cramer, after an exhaustive study of the

subject, concludes that there is no typical value for moisture and ash; both depending upon conditions of growth.

As regards the ash, Cramer, in some very interesting work on the cholera bacillus, finds that in this germ it depends within certain limits, both qualitatively and quantitatively, upon the culture medium. For instance, by using media rich in phosphates or chlorids, the amount of ash can be increased to nearly 30 per cent. of the dry substance. He also notes that the carbon and hydrogen are fairly constant for all species, not far from 51 per cent. and 7 per cent. respectively; while the nitrogen may vary widely, from 5.34 per cent. to 11.15 per cent. in *subtilis* as found by Vincenzi, and to 16.2 per cent. in anthrax as reported by Dyrmont. His observations of the effect of increasing the peptone in the medium, and of using grape sugar, show that bacteria adapt themselves to the culture medium, especially as regards their proteid constituent. Thus, comparable results can be obtained only from cultures of similar species, at the same stage of development and grown under like conditions.

Lyons has observed a certain relation between the carbohydrate formation and the carbohydrate in the medium. Thus, with increase of grape sugar in the medium, he found in the germ less proteid and more substance extracted by alcohol and ether.

Rodet calls attention to variations in chemical properties in the same species due to variations in the media, in temperature, light, etc. Variations in the indol reaction of the colon bacillus afford a familiar example. He states further that the pigment of *Bacillus pyocyaneus* depends upon the amount of oxygen, and in albumin cultures is green, in peptone cultures blue; that the virulence of the cholera vibrio, which has been diminished by laboratory culture, can be increased by growth on gelatin containing phosphate or a trace of iron.

Lepierre finds α -glucoproteins having the general formula $C_nH_{2n}N_2O_4$ ($n=6-11$) especially adapted to furnish nitrogen to microorganisms. Of forty-five different organisms studied, most used any glucoprotein; but diphtheria, tetanus, and some others preferred those containing eight or nine atoms of carbon; while some, tuberculosis for example, preferred those with

higher carbon content. However it is not surprising that organisms sufficiently sensitive to be used in separating stereoisomers should respond to slight changes in external conditions.

PROTEIDS. The earliest separation of proteid from bacteria was the isolation of mykoprotein from putrefying bacteria by Nencki and Schaffer in 1879. Later Nencki attempted to prepare mykoprotein from *Bacillus anthracis*, but obtained a new body, which he called anthrax protein. Brieger found in Friedländer's pneumococcus a proteid which contained less nitrogen than does mykoprotein. Lewith reports that Hellmich obtained from hay bacilli a substance with the properties of a globulin. Buchner obtained proteids from many different species of bacteria by extracting with water, with dilute alkalies, and also with 40 or 50 per cent. glycerin. Krehl and Matthes digested bacteria of various strains with pepsin, and isolated deuterioalbumose according to Neumeister's method. Brieger and Fränkel, Wasserman and Proskauer, and Dzierzgowski and Rekowski, all have reported toxalbumins from diphtheria cultures. Murillo also reported a toxic proteid from the same germ. Hammerschlag obtained from tubercle bacilli a proteid which he believed to be the source of the acid-fast character; but as the germs were also extracted with alcohol and ether, his reasoning would apply as well to the fatty constituents as to the proteid. Von Hoffman reported six proteids, and Weyl reported a toxomucin from the tubercle bacillus. Nitta obtained from crude tuberculin an albumose which acts like tuberculin, but is five times as active. As the specific action is destroyed neither by sodium nitrite, formaldehyde, nor by hydroxylamin, he concludes that it is not due to a labile amido, aldehyde, or ketone group. From *Bacillus tuberculosis* Ruppel obtained tuberculosamin, a body having the properties of a protamin.

NUCLEIN AND RELATED COMPOUNDS. From their action toward basic anilin dyes, Dreyfuss argued the presence of nuclein in all bacteria; Gottstein advanced as additional proof, their behavior with hydrogen peroxid, and the presence of phosphorus. Vandelde reported finding nuclein in *Bacillus subtilis*. Dietrich and Liebermeister found microchemical evidence that granules in old bacilli contained nuclein. Nishimura separated nuclein bases from a water bacillus grown on potato. Lustig and

Galeotti reported a nucleoproteid from the pest bacillus. From an organism resembling *Bacillus ranicidus*, Galeotti obtained a nucleoproteid having 11.99-12.21 per cent. of nitrogen, and 0.94-1.16 per cent. of phosphorus. From anthrax bacilli, Casa-grandi, Galeotti, Paladino, and Tiberti prepared nucleoproteids for immunity experiments. Ruzicka regarded nuclein as the chief constituent of this germ. Aronson obtained from the bacillus of diphtheria, one product which gave proteid tests, and upon decomposition yielded xanthin bases, pentose, and albumin. A second substance from the same bacillus yielded xanthin bases, pentose, and phosphate. Blandini reported nuclein and nuclealbumin from the typhoid bacillus. Enea obtained from both typhosus and subtilis a toxic nuclein. From the bases and phosphorus in extracts of *Sarcina lutea*, Wheeler found evidence of a nuclein body in that germ. From several different germs Iwanoff reported nucleoproteids which gave the biuret reaction, and contained about 16 per cent. of nitrogen, 2 per cent. of phosphorus, 2 per cent. of sulfur, and 2 or 3 per cent. of ash. Certain kernels found in bacteria, Meyer regards as reserve material consisting of nucleoproteid with a relatively large amount of nucleic acid derivatives.

Several investigators have found nucleoproteids in *Bacillus tuberculosis*. Klebs reported a nuclein containing 8-9 per cent. of phosphorus; de Schweinitz a nuclealbumin; while by a pressure of 400-500 atmospheres upon the moist germs, Hahn obtained a relatively large amount of nuclealbumin. Ruppel separated a nucleic acid containing 9.42 per cent. of phosphorus, which he designated as tuberculinic acid. Levene reported from the same species, three proteids, nuclein, tuberculinic acid, and crystals which he regarded as a mixture of thymine and uracil, also cytosine. Kutscher, Wahlen, and Trudeau, Baldwin and Kinghorn also reported nucleo-compounds from this germ. Kitajima described several different toxic substances, all of which contain tuberculinic acid (Tuberkelthyminsäure).

Wheeler has analyzed a phosphorized glycoproteid from *Bacillus typhosus* with the following percentage composition: C-48.27, H-6.28, N-10.92, S-1.45, P-0.403, and a trace of iron. Vaughan considers the colon germ as a chemical compound, a glyconucleoproteid. Lepierre and Charrin and Desgrez

report mucin from different germs; while Rettger states that he has found it as a product of many species of bacteria, independent of the presence of carbohydrate in the culture medium.

CARBOHYDRATES. Scheibler, and Smith and Steel reported gums from the viscous growth of bacteria in sugar juice. Schar-dinger, Ward and Green, and Kramer obtained carbohydrates from bacterial slime. From *Bacillus subtilis* Dreyfuss obtained a substance which reduced Fehling's solution, and from which he obtained glucosazone crystals. This he regarded as evidence of cellulose, but as Vincenzi failed to find it in the same germ by other methods, Dreyfuss's results were probably due to some other carbohydrate. Hemicellulose was found by Nishimura in a water bacillus, in *prodigiosus*, and in *Staphylococcus pyogenes*. De Schweinitz and Dorset found evidence of a small amount of cellulose in the tubercle bacillus, but not in the bacillus of glanders; Hammerschlag also reported cellulose in the tubercle germ; and Brown obtained it from the membrane of *Bacterium xylinum*. Wheeler found in yellow sarcine two carbohydrates, but no evidence of cellulose.

Both Bendix and Aronson obtained pentose from bacteria. Iwanoff, Emmerling, Helbing, and Bulloch reported chitin from germ substance. Levene has obtained glycogen from *Bacillus tuberculosis*.

FAT, WAX, ETC. In the earlier investigations of the composition of bacteria, alcoholic and ethereal extracts were often assumed to be fats without further investigation. Cramer, Meyer, and Klebs, all isolated fatty substances from germs. Nishimura found various fatty acids in a water bacillus, and isolated 0.68 per cent. of the dry germ substance as lecithin.

From the tubercle bacillus Hammerschlag reports obtaining fatty acids which melt at 63°C ; de Schweinitz and Dorset have isolated several acids, one of which was crystalline, melting at $161\text{--}164^{\circ}\text{C}$., and having the composition corresponding to the formula, $\text{C}_7\text{H}_{10}\text{O}_4$. Ruppel and Aronson have obtained wax as well as fat; that is, esters of acid and high alcohol, as well as esters of acid and glycerin. Levene finds that this wax melts between 55° and 60°C ., its analysis corresponds to the empirical formula $\text{C}_{12}\text{H}_{24}\text{O}_3$, and it is not saponified by ordinary methods.

Bulloch and MacLeod have isolated a pure alcohol which retained stain after immersion for eight days in 25 per cent. sulfuric acid. Klein finds that the young bacilli are not acid-fast, but thinks that property due to chemical substances which they produce in later stages of growth; while Marmorek regards the young germs, containing very little wax and fat, as the ones concerned in the tuberculin reaction. Dorset and Emery also refer the characteristic staining properties of this germ to higher alcohols of the aliphatic series. Kresling reports 38.95 per cent. of fat, 0.16 per cent. of lecithin, and some cholesterin from the tubercle bacillus. MacDonald calls attention to the resemblances between wool fat, hydrous cholesterin, and the fat of this germ.

Beebe and Buxton found the pellicle on a growth of *Bacillus pyocyaneus* to consist of masses of bacteria and bundles of fatty crystals. Lipochromes and other pigments have been reported as integral parts of the bacterial cell. Thus, Jordan obtained a blue pigment from *B. pyocyaneus*. Detweiler separated pigments from *B. violaceus* and from *prodigiosus*, and gives a summary of the literature of bacterial pigments.

CELL PLASMA. Hahn mixed bacteria with sand and subjected them to a pressure of 400-500 atmospheres. He thus obtained a clear liquid, which contains much coagulable albumin, shows reducing action, gives the ordinary proteid reactions, and undergoes autodigestion less readily than yeast plasma. Macfadyen and Rowland obtained cell plasma by rubbing up dry germs in a mortar submerged in liquid air, moistening the mass with physiological salt solution, and centrifugating. This gives an opalescent solution of the intracellular constituents. One cubic centimeter from typhoid germs, dried at 100° C., gave 0.07174 gram of substance, of which 0.027 gram was fixed ash. The residue contained 6.914 per cent. of nitrogen and 37.64 per cent. of ash. The plasma of typhoid, streptococcus, and several other bacteria was found highly toxic. Various species of bacteria were kept for six months immersed in liquid air without impairing their vitality. Bassenger and Mayer also froze and pulverized germs, getting a plasma which gave proteid reactions, but gave negative results with Bial's pentose reagent and Fehling's solution. Maasen found plasma to show

reducing action, and thinks there are two reducing substances in the cell, which are very labile, resembling ferments.

ENZYMES. Emmerich and Löw find that pathogenic bacteria produce proteolytic enzymes which dissolve the nucleoproteid of the bacterial cell, and for which they suggest the name nuclease. The nucleases give oxygen with hydrogen peroxid, dissolve thymol-gelatin, egg albumin, and fibrin. Some render bacterial poisons non-toxic. In dry form they are quite stable; a solution can be made more stable by the addition of dextrin. They unite with albumins to form complex bodies of high molecular weight which are said to be very efficacious in conferring immunity. Pyocyanase, a nuclease from *Bacillus pyocyaneus*, is a yellowish green, amorphous powder, dissolving in water to form an alkaline solution. Ferrocyanic acid does not precipitate it, nor does it give the Millon and biuret tests. It is stable at 100° C., and is said to resemble trypsin. Both Dietrich and Petrie think the effects ascribed to pyocyanase to be due to other causes; to alkalinity, to action of poisonous organic bases, to starvation, or to change in osmotic pressure. Fermi also believes that bacterial enzymes can not digest the bacterial cell.

Rogers, Malfitano, Abbot and Gildersleeve, Savage, Grau, Jones, Eijkman, Levy and Pfersdorff, and Mavrojannis, all find evidence of enzymes in bacteria, some precipitating casein, some liquefying gelatin, others, hemolytic, amylolytic, proteolytic; but they do not give chemical data.

Rettger grew bacteria of various kinds upon cooked potato, scraped off the growth, mixed it with physiological salt solution and either toluol or chloroform. The mixture was tested from day to day with alkali and copper sulfate, the intensity of the color showing the progress of digestion. With the colon germ autolysis was slow; while with prodigiosus, autolysis was complete in 10 or 12 days, no proteid being left. Among the decomposition products were considerable leucin, little tyrosin, basic products considerable in amount but not enough for identification, and phosphoric acid. Young and freshly prepared mixtures were free from the odors characteristic of autolysis, and gave little leucin or other bases. With *B. pyocyaneus* autolysis was even more rapid, the proteid completely disappearing in four or five days. The author believes that auto-proteolysis goes on in

living cultures, and is a cause of irregularities in staining. He also suggests that diphtheria toxin may be intracellular, and may be liberated in this way. He finds autolysins sensitive to antiseptics, toluol being more active than chloroform.

TOXINS. Brieger obtained from tetanus cultures tetanin, $C_{13}H_{30}N_2O_4$, and tetanotoxin, $C_5H_{11}N$, well characterized poisonous compounds giving crystalline salts. He also reported typhotoxin, $C_7H_{17}NO_2$. Recently, however, little attention has been paid to these compounds, and Vaughan and Novy say that they are not to be regarded as the true toxins of these germs. Bertarelli found the cell substance of *Bacillus prodigiosus* fatal to various animals, while the soluble products were only slightly toxic. J. W. Vaughan reports an endotoxin from the anthrax bacillus, and gives an abstract of the literature upon the subject. Kolle gives a good discussion of the distinctions between extracellular toxins and endotoxins. He, Yersin, Calmette and Borrel, Pfeiffer and Dieudonné, all agree that the toxin of pest is intracellular, while Markl, and Kossel and Overbeck regard it as extracellular. Todd thinks the bacillus of dysentery contains endotoxin, as the toxin is found in young cells and in old cultures. Lüdke holds that the poisons found in filtrates from typhoid and dysentery cultures are endotoxins, set free by autolysis. Conradi reports that these toxins are weakened by antiseptic autolysis, and even by aseptic autolysis if continued longer than forty-eight hours. From streptococcus both Aronson and Bonome report an endotoxin, which Marmorek says belongs to the diastase group. Simon finds in the same germ a relatively weak endotoxin, and a much stronger extracellular poison, the secretion of these two being independent of each other. Kossel reports a toxin formed in the cell, while Gelston finds both extracellular and intracellular poisons in the diphtheria bacillus, cultures giving the most powerful extracellular toxins not giving the most toxic germ substance. Neisser and Wechsberg report two poisons from staphylococcus, having different haptophore and toxophore groups. From the tubercle bacillus Maragliano extracts a toxin by means of water, and Sciallero one by means of olive oil and ether. Rodet, Lagriffoul, and Wahby after several years of investigation, conclude that typhoid cultures are more toxic than the germ, that the toxin is not a necessary constituent

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of the germ, is not an endotoxin, but a secretion. Kiessling gives a *résumé* of work upon the toxicity of the colon germ. Tichomiroff finds that nucleic acid precipitates tetanus and diphtheria toxins. Murillo reports toxin in diphtheria protoplasm, and that the toxicity of diphtheria cultures has two maxima, one in the first week and another in the third week. Charin separates from the toxin of *B. pyocyaneus*, a volatile constituent, another soluble, and a third insoluble in alcohol, each having a different physiological action.

Brieger and Boer report toxins from tetanus and diphtheria which do not give proteid and peptone tests, but seem nearly related to acids. Baldwin and Levene, however, report that both these toxins are digested by proteolytic enzymes, and so infer their proteid nature. Since they found tuberculin destroyed by trypsin but merely weakened by pepsin, they argue that it is related to nucleoproteids. Kinghorn succeeded later in rendering tuberculin inactive by six to ten days' peptic digestion. Belfanti thinks diphtheria toxin a true nucléin. Hayashi has obtained a preparation which he regards as a double compound of tetanus toxin and basic zinc carbonate. He considers the toxin a proteid, possibly a primary albumose. Brieger and Boer report that certain zinc salts precipitate tetanus and diphtheria toxin quantitatively, but that the compound contains no trace of albumin or peptone. By similar methods Brieger and Kempner obtain toxin from *B. botulinus*. Baudran subjected various toxins to the action of calcium permanganate, and found the products similar to those formed from strychnin under the same conditions. Brieger reports a toxin from *staphylococcus* which gave the alkaloidal reactions. Gautier suggests that the toxins usually consist of an alkaloidal substance joined to a very active nitrogenous body. Baldi thinks that in diphtheria cultures toxin replaces sulfur in the proteid molecule. Austin obtained toxin from typhoid and from diphtheria germs, and suggests that these toxins may be united to albumose much as iron or phosphorus is bound in caseose or casein. Auclair extracted the cell substance of various species of bacteria with ether or chloroform, and obtained products which showed the toxic action of the germs from which they came. He concludes that a study of the toxins is more important than the life history of the germ,

and that these extracts may serve for the identification and differentiation of germs, and as possible producers of immunity. Wechsberg regards the toxins as receptors of bacteria, a part of the cell, and finds that immunization *in vitro* increases the throwing off of these side chains; by growing diphtheria germs in bouillon containing antitoxic serum he increased the toxicity tenfold. Zangger calls attention to the great variations in their chemical properties shown by the toxins; most, however, are colloids and basic, some lose their toxicity by the action of solvents. By using a dialyser in which the tension of the membrane can be varied, van Calcar finds evidence that diphtheria toxin has a lower molecular weight than toxon, and toxon than peptone. Römer ascribes van Calcar's results to the filling of the pores of the membrane, although he had reached similar conclusions by quite different methods. Hailer and others agree that diphtheria toxin has a lower molecular weight than antitoxin. Biltz, Much, and Siebert find in the neutralization of toxin, that the toxin is first bound to the antibody by adsorption, following the general rules of colloids, and then this compound decomposes more rapidly than the free toxin. Gay thinks that toxins enter into direct combination with the protoplasm of the cell. Werner finds oxygen necessary to the germination of many bacilli, while on the other hand it destroys the toxin secreted. This perhaps explains the lack of virulence in artificial cultures.

By means of an alcoholic solution of sodium hydrate, Wheeler has split off from the colon bacillus an exceedingly virulent poison, which is soluble in water and in alcohol, gives the ordinary proteid color reactions, but does not yield a reducing carbohydrate. She finds no evidence that it is alkaloidal. It is broken up by concentrated acid, giving a toxic crystalline compound. Vaughan regards the poison obtained by this method the specific toxin of the colon germ, containing both haptophore and toxophore groups. He gives chemical and physiological reasons for suspecting the toxophore group to be a neurin-like body. In this connection it is interesting to note that Kyes and Sachs find a lecithin group necessary to the hemolytic action of cobra venom, and Kyes suggests that cholin may be the toxophore group of lecithin. Wolff thinks endotoxins identical with the albu-

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minous substance of the cell, and their action that of any other foreign proteid. Foreign proteids do not cause the formation of antibodies, and this is the great distinction between endotoxins and toxins. Vaughan thinks the formation of endotoxins a synthetic process, not essential to the life of the cell, nor are they the sole constituents of the cell plasma.

Perhaps no recent chemical problem has attracted more widespread attention than the application of the methods of physical chemistry to the problems of physiological chemistry, and especially to the neutralization of toxins by antitoxins. Arrhenius, Madsen, and others find experimental evidence that it follows the law of mass action; Ehrlich, Sachs, Nernst, and others hold that the action is not reversible, and is much more complicated.

IMMUNE BODIES. Agglutinums, precipitums, and hemolysins have been split off from the bacterial cell, but their study has been physiological rather than chemical. Pick, who gives an extensive bibliography, has made an exhaustive study of the substances from typhoid germs which react with typhoid immune serum. He split off from the germs an active substance which is not ordinary proteid, and which burns with an odor like that of subliming leucin. Madsen, Weingeroff, Bulloch and Hunter, and Kayser are among those who have investigated bacterial hemolysins. Buxton gives a very clear outline of the present theories of immunity, and describes the action of bacteriolysins as directed not against the bacillus as a whole, but against certain albumins in the germ cell. The intermediary body has affinity for these albumin molecules and unites with them; the complement unites with the intermediary body and can then by virtue of its enzymotic nature dissolve or destroy the albumin molecules to such an extent that the bacillus is destroyed. He adds that as it is generally accepted that no antibodies are formed against compounds simpler than albumins, this raises the question whether endotoxins are albuminous or simpler.

EXPERIMENTAL.

MATERIAL. The material used for this work is *Bacillus coli communis* obtained from normal feces, and made more virulent by repeated passage through animals. The large copper tanks in use in this laboratory were filled with ordinary 2 per cent.

agar medium, sterilized, and inoculated with virulent cultures of the colon germ. After seven to fourteen days, the growth was scraped from the agar by means of bent glass rods, a very little water added if necessary, and the germs drawn off into flasks by suction. The cell substance was precipitated by pouring into 95 per cent. alcohol. It was filtered, repeatedly washed with alcohol, extracted with ether in a Soxhlet, dried first between filter papers, and then *in vacuo* over sulfuric acid. When thoroughly dry the germs were pulverized in a porcelain mortar, then in an agate one, and passed through a fine sieve. In this way a light gray or cream-colored powder was obtained which is very nearly pure cell substance without the fat.

MOISTURE AND ASH. The cell substance thus prepared takes up moisture readily and holds it tenaciously, but may be dried to constant weight by heating small amounts in a steam-drying oven for many days at from 85° to 95° C. If the temperature runs down to 60°, it may absorb moisture even in the oven. One sample was heated to 105° during working hours for two or three days, and kept in a desiccator during the intervals. Under this treatment it steadily increased in weight. Drying *in vacuo* over sulfuric acid is on the whole the most satisfactory method, although it requires many days or even weeks.

The dried cell substance burns with a flame, forming volatile and liquid products, giving off odors characteristic of nitrogen compounds, and finally leaving a greenish ash. Two determinations gave the following results:

- 0.346 gm. of substance gave 0.0296 gm. of ash, or 8.55 per cent.
- 0.496 gm. of substance gave 0.0431 gm. of ash or 8.68 per cent.

Values reported for other germs vary from 3 per cent. in putrefactive bacteria to 13 per cent. in prodigiosus, or, by using special media, to nearly 30 per cent. in the cholera bacillus; while in the tubercle bacillus the ash has been found to vary from 1.77 per cent. to 5.92 per cent. according to conditions. The ash from the colon bacillus contained sodium, potassium, phosphates, and small amounts of calcium, aluminum, and copper. A slight residue insoluble in acid was probably silica. Sulfates and chlorides were not found. In comparison with the data from other germs, these findings are noteworthy only in the absence of magnesium, and for the presence of copper and

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aluminum. Presumably the copper comes from the tank, and it is very likely that the aluminum comes from the agar.

Phosphorus was the only constituent of the ash quantitatively determined.

The ash was dissolved in nitric acid, the phosphate precipitated with ammonium molybdate, dissolved in ammonia, reprecipitated with magnesia mixture, and weighed as pyrophosphate. The following results were obtained:

Weight of Sample	Weight of Pyrophosphate	Weight of Phosphorus	Per cent. of Phosphorus
0.496 gm.	0.0475 gm.	0.01323 gm.	2.68
0.346 "	0.0380 "	0.01059 "	3.06

The mean of these determinations, 2.87 per cent., agrees quite closely with Levene's finding, 2.67 per cent. of phosphorus in the tubercle bacillus from mannite cultures. Most observers report smaller results, but it should be noted that my samples are free from fat and wax, and therefore the percentages are higher than if calculated for the whole germ.

In view of the number of elementary analyses of different bacteria already reported, and more especially in view of the wide variations in composition caused by the nature of the nutrient medium, the stage of development of the organism, the temperature and other conditions of growth, it did not seem worth while to make determinations of carbon and hydrogen.

PRELIMINARY WORK. Some earlier investigations in this laboratory upon the toxicity of the colon germ showed the desirability of studying the action of dilute acid upon the cell substance. Accordingly samples were treated with 1 per cent. sulfuric acid under varying conditions. On filtering, a light brown or straw-colored liquid was obtained. This readily reduced nitric acid, and gave the typical xanthoproteic color on the addition of ammonia. In no case was there more than a slight biuret test, and there was too much sulfate present for a satisfactory Millon test. The α -naphthol test for furfurol was positive. Alcohol gave a voluminous precipitate, A, which will be described more fully under another heading. The alcoholic filtrate, B, was neutralized with sodium hydroxid, the sodium

sulfate filtered out together with some organic matter mechanically carried down, and the liquid distilled under diminished pressure at 30°–35° C. The liquid residue, C, left after distillation, turned yellow on heating with potassium hydrate, but gave neither the biuret nor Millon's test. Again, the xanthoproteic and α -naphthol tests were positive, but it failed to reduce either Fehling's or Nylander's solution. It yielded precipitates with ammonium molybdate, phosphomolybdic acid, ammoniacal silver nitrate, and picric acid. A guinea-pig was injected with 5 c. c. of C with no apparent effect.

As there were indications of organic bases, Residue C was tested for xanthin bases in the following manner: on the addition of barium hydrate and barium chlorid it gave a precipitate, D, and a filtrate, E. Precipitate D was warmed with dilute sulfuric acid (1 to 4), and the resulting solution was found to contain carbohydrate, phosphate, and bases. After removing the phosphate, the bases were precipitated by ammoniacal silver nitrate. The silver compound was decomposed by hydrochloric acid. The acid solution gave a gelatinous precipitate with ammonia, soluble in excess of the reagent. Filtrate E was treated with carbon dioxid to remove the barium, and the excess of carbon dioxid removed by boiling. The resulting solution gave tests for furfural and for bases. It was concentrated to about one-fourth its volume, and precipitated with ammonia and ammoniacal silver nitrate. This precipitate was dissolved in boiling nitric acid, specific gravity 1.1, and allowed to stand forty-eight hours, but no crystals corresponding to the hypoxanthin fraction were found. Accordingly the solution of the silver compounds was concentrated, decomposed with hydrogen sulfid, filtered, the filtrate evaporated, and treated with ammonia. A precipitate was obtained which suggested xanthin. The silver sulfid precipitate was worked up for guanin, which might be carried down with the sulfid, and a small amount of an organic precipitate was obtained. Thus there were indications of the presence of at least two of the xanthin bases, although the amounts were not sufficient for identification. It may be added that larger amounts were obtained as by-products in the work on hexon bases to be described later, and these indications confirmed.

In another set of experiments, Residue C was treated with hot

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concentrated barium hydrate until nearly neutral, distilled with magnesia to remove any ammonia formed, more barium hydrate added, and the sulfate filtered out. The filtrate was acidified and xanthin bases precipitated by silver sulfate. The solution, after removing the xanthin bases, was tested for hexon bases according to the method of Kossel and Kutscher, and found to give indications of lysin together with smaller amounts of histidin and arginin.

The residue, left after extracting the moist fresh germ with 1 per cent. sulfuric acid, was thoroughly mixed with 33.33 per cent. acid, and boiled for eight hours in a flask with a reflux condenser. The black extract was filtered, and barium hydrate added until the greater part of the acid was neutralized. After removing the barium sulfate, the filtrate yielded no precipitate with alcohol did not give the biuret test, nor did it reduce Fehling's solution. With nitric acid there was vigorous action, and the addition of ammonia brought out a faint xanthoproteic color. Here again the appropriate tests gave indications of both xanthin and hexon bases.

The residue, after repeated extraction with dilute sulfuric acid (from 1-5 per cent.), was treated with 2-4 per cent. sodium hydrate either upon the water-bath or over a free flame. In every case the substance went into solution readily, leaving only a slight coating on the filter. The slight residue gave no proteid test, contained no nitrogen, but gave test for carbohydrate. In one case it was removed from the filter, and the organic matter approximately determined. The total residue was about 0.4 gram, equivalent to 0.8 per cent. of the cell substance used. The organic matter was only 0.15 gram, equivalent to 0.3 per cent. of the original.

The alkaline extract was neutralized with hydrochloric acid and then poured into 95 per cent. alcohol. A light-colored, flocculent precipitate (G) was obtained. This turned dark on the exposure to air incident to filtration. It was twice dissolved in 0.5 per cent. potassium hydrate and reprecipitated. Each time the fresh precipitate was white or nearly so, but the utmost care in filtering, even in an atmosphere of carbon dioxid, did not prevent its turning dark. The solution in alkali gave the xanthoproteic and furfurol tests, but neither the biuret nor

Millon's test. Copper chlorid gave a precipitate, but picric acid and platinum chlorid did not. The solution was accordingly acidified with picric and acetic acids, copper chlorid added, and the mixture poured into three volumes of alcohol. An aqueous solution of the precipitate thus obtained did not reduce Fehling's solution, but after boiling with hydrochloric acid it reduced both Fehling's and Nylander's solutions, and also gave the furfurol test, thus showing the presence of a carbohydrate. Precipitate G burned readily, puffing up and glowing as does nucleic acid, then fusing and leaving a dark ash. Two determinations of phosphorus gave the following:

Weight of Sample	Weight of Pyrophosphate	Weight of Phosphorus	Percentage of Phosphorus
0.4723 gm.	0.0524 gm.	0.0145 gm.	3.09
0.6469 "	0.0685 "	0.01895 "	2.93

As the amount of carbohydrate was not determined, this simply shows that the nuclein body present contains more than three per cent. of phosphorus. Its behavior, however, points toward nucleic acid.

The work thus far described was merely preliminary, and, like the preliminary tests in ordinary analysis, has shown the action of various solvents sufficing to bring the whole into solution; then to each partial solution a large number of tests (many of those giving negative results not reported in this brief account) were applied to show what classes of compounds might be sought. The amounts used were too small to permit the purification and identification of the compounds obtained, but indications of proteid, carbohydrate, nucleo-compounds, purin and hexon bases have been found. It will be remembered that the fat and wax had been previously removed from the material. From the great number of problems suggested by this preliminary work, two will be reported upon in this paper: a study of the alcoholic precipitate previously designated as A, and the isolation of lysin. While no attempt has been made to isolate the carbohydrate, the abundance of furfurol indicates that pentose, reported in other germs, is present in the colon bacillus as well. No evidence of cellulose was found. In this connection it may

be remarked that in some of the earlier literature the nitrogenous matter was assumed to be simple proteid, and the excess of carbon assumed to give the content of cellulose. Whether the bacterial cell does or does not contain a nucleus, it certainly contains nucleic compounds, and their abundance adds interest to the theory that these cells are mainly nucleus. Indeed, the almost monotonous similarity shown by the extracts prepared under widely varying conditions is very suggestive, especially in conjunction with the lack of differentiation revealed by microscopic study, and with the theory of the bacterial cell as a chemical unit recently advanced by Dr. Vaughan.

A CLEAVAGE PRODUCT. In the preliminary work above described, a cleavage product split off by very dilute acid was deemed worthy of further investigation. Accordingly, the pulverized cell substance after extraction with alcohol was treated with about forty times its weight of 1 per cent. sulfuric acid. Some samples were shaken at ordinary temperatures, while others were heated on the water-bath. The most satisfactory results were obtained by rubbing up the cell substance in a mortar with a little of the acid, then adding acid little by little, with constant stirring, and heating on the water-bath for an hour. On standing, a heavy deposit settled, leaving an opalescent fluid. Various expedients for filtering were tried, a folded hard filter proving the best. With this the first few cubic centimeters come through turbid, but soon the pores of the paper are filled, and the filtrate becomes a clear golden brown. However, the filter gradually becomes clogged, and filtration proceeds more and more slowly. A suction pump is no help, for the pores of the paper are very soon so filled as to stop filtration completely. At best, five days were required to filter two liters.

The filtered acid extract was poured slowly, with constant stirring, into from three to five volumes of 95 per cent. alcohol. The resulting precipitate was white, amorphous, flocculent, and at first very voluminous. This was allowed to settle several hours, filtered, and washed with alcohol until the washings were no longer acid. Prepared thus, the precipitate dissolves in a volume of water equal to the original volume of extract, giving an opalescent solution which filters readily leaving no residue. The longer the precipitate stands in contact with

alcohol, the less readily it redissolves; on the other hand, unless it completely subsides, filtration is slow. The aqueous solution was reprecipitated by pouring into three volumes of absolute alcohol, allowing to settle as before, and filtering as rapidly as possible, because while moist it darkens on exposure to the air. Here again a filter pump was of no assistance, and filtering in an atmosphere of carbon dioxid was not satisfactory. The precipitate was dried between folds of filter paper, and then *in vacuo* over sulfuric acid. It diminished very greatly in bulk on drying, was then hard and horny in texture and gray or brown in color, but when thoroughly pulverized was lighter.

The opalescent solution is acid to litmus, and becomes clear and transparent on the addition of a trace of alkali. It gives a well marked xanthoproteic test, the other color tests for proteid only very slightly. Both phosphotungstic acid and silver nitrate give precipitates, while albumin and ammoniacal silver nitrate do not; no free phosphate ions were found. It gives a good furfural test with α -naphthol, but does not reduce Fehling's solution either before or after boiling with hydrochloric acid.

The cleavage product, like the cell substance, has great affinity for moisture. After standing six months in a bottle with a glass cap, samples absorbed water while being weighed in a watch glass. Small samples (0.5 gm.) dried to constant weight if kept at 98° C. for seven or eight days, or if kept *in vacuo* over sulfuric acid at room temperature for ten days. Larger amounts kept for a few days at 98° C., and then between 105° and 110° C., required still longer, and sometimes showed signs of decomposition before becoming constant.

This substance liquefies on burning, gives off volatile products, and the odor of burning feathers, finally leaving a dark residue. Determinations of the ash are not satisfactory, for particles of organic matter fuse into the residue and can not be consumed. Hammarsten says the nucleins give an acid coke containing metaphosphoric acid which is burned with great difficulty. Very likely this is one trouble here. Further, in trying to burn the organic matter out of the liquid mass, some of the mineral constituents are volatilized. An average of several determinations gives 30.74 per cent. of fixed ash. The amount of inorganic

matter is probably even higher, and includes the metals present in the organism as integral parts of the cell, as well as those taken up from the alkaline culture medium and from the tank. All these would be seized with avidity by the acid used in the extraction, and the sulfates thus formed would appear in the alcoholic precipitate. The ash is not wholly soluble in either nitric or hydrochloric acid. It contains both sulfate and phosphate.

Nitrogen was determined by the Kjeldahl method; sulfur by fusion with potassium hydrate and potassium nitrate, and precipitation as barium sulfate; phosphorus was determined in the ash by the molybdate-magnesia method. A comparison of the results of these determinations with figures obtained from the cell substance is given in the following table.

TABLE III.
COMPARISON OF CELL SUBSTANCE AND CLEAVAGE PRODUCT.

	Ash. Per cent.	Nitrogen. Per cent.	Phosphorus. Per cent.	Sulfur. Per cent.
Cell substance,	8.61	10.65*	2.87	None in ash.
Cleavage product	30.74	6.49	5.30	9.22
Do., ash free	—	9.40	—	—

* Air-dried samples not at constant weight were used for this determination.

This substance proves to be toxic, somewhat more toxic, weight for weight, than the whole germ. From a number of animal experiments, the following are reported as typical: One hundred milligrams of the substance were dissolved in 10 c.c. of sterile water to which one drop of sodium hydrate solution was added. Guinea-pigs were injected intra-abdominally at 11:20 A.M., as follows:

No. 1	received	5 c. c.,	corresponding	to	50 mg.	of the substance.
" 2	"	2.5 "	"	"	25 "	"
" 3	"	1 "	"	"	10 "	"
" 4	"	0.5 "	"	"	5 "	"

No. 1 was practically dead at 5 P.M. the same day, although it did not actually draw its last breath until after six. No. 2 died at six P.M., while Nos. 3 and 4 were found dead at 7 o'clock

the next morning. The post-mortem findings were the same as those reported by Vaughan as characteristic of the colon toxin.

Extracts obtained by heating with acid in an autoclave at 140° – 144° C., or by heating the moist fresh germ with acid on a water-bath for two hours, gave precipitates with alcohol which were markedly less toxic. One hundred milligrams of each of these precipitates, dissolved and injected as in the previous experiments, made the animals very sick for a time, but they finally recovered. No chemical differences have been found between the more toxic preparations and the less toxic ones. Behring found tuberculinic acid prepared by extracting the germ with neutral salts or dilute alkali to be five times as toxic as preparations of the acid obtained by boiling the germ with barium hydrate. Levene's experiments showing the instability of tuberculinic acid have already been cited. The variations in toxicity in these preparations from colon and tubercle bacilli may be due to slight differences in chemical composition, or to the instability of the compound and the consequent decomposition of amounts varying with the conditions.

There is abundant evidence of the instability of some of the bacterial toxins. Madsen, for example, found that a 0.1 per cent. solution of tetanolyisin in physiological salt solution was weakened by standing an hour at room temperature. Markl had accumulated a quantity of pest toxin for use, and one hot day the temperature of the laboratory was so high as to spoil it. He says that body temperature is unfavorable for this toxin, and suggests that at this temperature toxic substances are formed which in the nascent state unite with the albumin molecule to form an innocuous compound; accordingly in a host this toxin does not accumulate. On the other hand, it has been shown that heating the colon germ with water in an autoclave to 154° C., or in a sealed tube to 184° C., does not destroy its toxicity. Also Sluyts finds cholera toxin, with a chemical nature much like that of the colon toxin, to be quite stable. It can be heated to 120° C. for an hour, or an hour and a half, or exposed to direct sunlight for twenty-four hours, without losing its toxicity.

It seems probable, however, that the toxicity of these colon preparations is due, not to the main body of the substance which responds to the chemical tests, but to the presence of

small amounts of an exceedingly virulent compound mechanically carried down. Wheeler's recent work on the colon toxin serves to confirm this view. Markl believes that attempts to isolate pure toxins fail because of their sensitiveness towards reagents, and also because of the tenacity with which they cling to the albumin molecule, whether acid albumin or abuminat. Brieger says also that if diphtheria toxin is precipitated by any organic or inorganic reagent for precipitating proteid, this simply shows the mechanical carrying down of the toxin by the proteid. Wasserman and Proskauer found toxin carried down by various precipitates, and suggest that the so-called toxalbumin may be a mechanical mixture of toxin and albumose. Roux and Yersin report that a precipitate of calcium phosphate in diphtheria bouillon is toxic, and think the toxicity is due to toxin which clings to the inorganic precipitate. Tichomiroff says that nucleic acid precipitates tetanus and diphtheria toxins.

Aronson, in his work on the diphtheria bacillus, found a great difference between extracts made from fresh material and from material that had been previously exposed to the action of alcohol and ether, and was able to make a distinction between the proteid and the toxin. Using 0.1 per cent. ethylendiamin, he extracted twenty times as much organic matter from fresh germ substance as from material which had previously been extracted with alcohol and ether; equal volumes of the two extracts, however, showed practically the same toxicity. He reasons that the bacteria are so hardened by the alcohol and ether that little proteid goes into solution, while the solubility of the toxin is only slightly affected. If the same ratio holds with these extracts from the colon germ, the animal experiment in which a preparation from the moist germ substance was used, should be compared only with injections of 5 milligrams of the preparations from germs previously extracted with alcohol, usually, but not invariably, killed within twenty-four which hours.

A recent article by Wolff suggests another but less plausible explanation. He regards the intracellular toxins not as an especial class of poisons, but thinks their action is that of any form of foreign albumin when introduced into a host (giftig wie jedes körperfremde Eiweiss). Bacterial proteids, then, are

to be considered denaturized endotoxins, their toxicity coming to light when they are brought into a soluble form that can be absorbed.

The residue, after extraction with acid at ordinary temperatures, was again treated with 1 per cent. acid under the same conditions. The extract was slightly colored, but contained too little organic matter to be worth working up. Again the residue, after extraction with acid on the water-bath, was thoroughly mixed with acid and heated in an autoclave at 138° to 144° C. for an hour. This extract was poured into alcohol, giving a small amount of precipitate, similar to the first, but darker in color and only slightly toxic. The residue from this second extraction was autoclaved for three hours at 140° to 144° C. with 1 per cent. acid, extracting little but humin substance. The residue from the third extraction was autoclaved at 140° to 144° C. for an hour with 5 per cent. acid, again to no avail.

Thus a single extraction at water-bath temperature splits off all that 1 per cent. sulfuric acid can remove from the germ, and it would seem that there is here a definite line of cleavage. The cleavage product insoluble in alcohol as prepared above is confessedly not pure, but even in its impure state permits the drawing of certain conclusions. It is not proteid, as it does not give the proteid tests except the ubiquitous xanthoproteic. The failure of the biuret test shows the absence of protamin. Tests show that it contains sulfate, as would be expected from its preparation; if its sulfur is all combined as sulfate, it contains 27.66 per cent. of SO_4 . On this basis, calculating percentages of nitrogen and phosphorus for compound free from sulfate, we have $\text{N}-8.98$; $\text{P}-7.33$. But these figures serve only to give minimal values. The high percentage of phosphorus and the failure of some of the color reactions exclude nucleoproteid and nuclein, but not nucleic acid. Having thus studied a product of cleavage obtained by gentle means, attention was turned to the results of more violent action by which crystalline products could be obtained in a pure state.

HEXON BASES. Since an open chain of six carbon atoms seems to be the favorite foundation for the carbohydrates found in nature, it is a striking coincidence, if nothing more, that

Kossel and others have found the same chain in so many forms of albumin. Moreover in animal metabolism much of the proteid is changed into carbohydrate, and, as Cohnheim suggests, this change can be most easily accomplished by oxidizing pre-formed chains of six carbon atoms. The preliminary experiments described above showed the presence of basic compounds, presumably hexon, in the cell substance of the colon germ. As one or more of these bases, lysin, arginin, and histidin, have been found in every proteid thus far examined, and in view of Kossel's theory that they are fundamental constituents of proteids, and thus a necessary part of the proteid molecule, and indeed that one of them is the nucleus of that molecule, their presence or absence in the bacterial cell is of considerable theoretical interest.

Lysin, $C_6H_{14}N_2O_2$, α - ϵ -diamido-caproic acid, $NH_2.CH_2.CH_2.CH_2.CH(NH_2).COOH$, the base to be especially considered here, was discovered by Drechsel in 1891 among the cleavage products of casein. Ernst Fischer, Siegfried, Kossel, Kutscher, Schulze and Winterstein, Abderhalden, and others have shown its wide distribution in all forms of proteid thus far examined for it, save gliadin, zein, mucedin, gluten fibrin, and certain protamins. Drechsel, Willdenow, and Lawrow prepared the dibenzoyl compound, to which they gave the name lysuric acid; Hedin separated lysin as a silver nitrate double salt; Herzog recommended the formation of a hydantoin by means of phenyl isocyanate; but the separation as picrate by the Kossel and Kutscher method is the one commonly employed. The xanthin bases are first removed by addition of silver nitrate to the acid solution. Histidin is precipitated by silver nitrate and barium hydrate to slight alkalinity; and arginin by excess of silver nitrate and barium hydrate. To the resulting solution phosphotungstic acid is added, precipitating lysin with cholin, betain, and some other compounds. The phosphotungstates are decomposed by barium hydrate, and lysin is precipitated as picrate.

For purposes of comparison, samples of lysin picrate and lysin chlorid were prepared from fibrin and from gelatin. Fibrin was subjected for some weeks to tryptic digestion following the methods of Kühne and Chittenden and of Kutscher. After the

removal of the undecomposed proteid, first by heat, then by successive saturation with ammonium sulfate in acid, in neutral, and in alkaline solution, lysin was separated as outlined above. Also gelatin was boiled for many hours with sulfuric acid, and lysin salts obtained identical with the corresponding preparations from fibrin.

Samples of the cell substance of the colon germ were boiled with acid, and the resulting solution treated by the Kossel and Kutscher method. In the first series of experiments, at every step in the process, indications pointed to the presence of all three of the hexon bases, but at the end, sticky hygroscopic substances were obtained which resisted all attempts to crystallize them. Picric acid gave a waxy or oily mass instead of well defined crystals of lysin picrate. Attempts to make the chlorid either directly or through the picrate, gave a syrupy, amorphous substance, exceedingly hygroscopic, which, after long drying *in vacuo* over sulfuric acid in the cold, was hard and somewhat brittle, but even in a cold room became sticky before it could be pulverized.

In two sets of experiments, in order to trace the decomposition of the proteid through its successive steps, frequent determinations of the amount of nitrogen in the solution were made by the Kjeldahl method. In the first, 173 grams of air-dried cell substance were boiled with nine times its weight of 33.33 per cent. sulfuric acid. This was filtered and the residue repeatedly boiled out with water until the washings gave only a slight precipitate with phosphotungstic acid. The extract and washings contained 18.37 grams of nitrogen, which is 10.62 per cent. of the cell substance used. The residue contained 0.659 gram of nitrogen, 0.38 per cent. of the cell substance. By addition there is then 19.03 grams of nitrogen in the sample used, or 11 per cent. of the germ. Thus the acid has brought into soluble form 96.53 per cent. of the total nitrogen. After precipitating the extract with phosphotungstic acid, there were left in the solution 9.65 grams of nitrogen. The phosphotungstic precipitate then contained 8.715 grams of nitrogen. Following the common assumption that phosphotungstic acid separates the monamido from diamido compounds, these results may be summarized as in Table IV.

TABLE IV.

DISTRIBUTION OF NITROGEN EXTRACT WITH 33.33 PER CENT. ACID.

	Weight in Grams	Percentage of Cell Substance	Percentage of Total N of Cell Substance	Percentage of N of Extract
Cell substance	173.			
N in extract	18.37	10.62	96.53	100.
N in residue	0.659	0.38	3.47	
Monamido N	9.655	5.581	50.73	52.56
Diamido N	8.715	5.04	45.8	47.44

In another series of experiments the nitrogen was determined at every step. Samples of air-dried cell substance gave 10.65 per cent. of nitrogen. One hundred grams of cell substance, weighed out under similar conditions, were extracted repeatedly with 5 per cent. sulfuric acid. In the extract 9.58 grams of nitrogen were found. Barium hydrate was then added, the sulfate filtered out and boiled with water several times, filtrate and wash water concentrated to the volume of the original extract, about five liters. This contained 8.10 grams of nitrogen; there were then 1.48 grams of humin nitrogen which boiling did not remove from the inorganic precipitate. After acidifying, adding silver nitrate, and filtering, there were left in the filtrate 7.639 grams of nitrogen; thus precipitating the xanthin bases had removed 0.464 gram of nitrogen. After precipitation with silver nitrate and barium hydrate, the solution contained 5.913 grams of nitrogen, 1.726 grams having been removed. After precipitation with phosphotungstic acid, the solution contained 1.704 grams of nitrogen. The silver precipitate for arginin and histidin was rubbed up with water containing sulfuric acid, and decomposed by hydrogen sulfid. To the resulting solution, silver nitrate was added, then barium hydrate to slight alkalinity, to separate the histidin from the arginin. The precipitate was decomposed as before with hydrogen sulfid, the resulting solution containing 0.454 gram of nitrogen. Phosphotungstic acid was added to the solution to separate the histidin from any thymine and uracil that might be present, and the precipitate decomposed by barium hydrate, giving a solution containing 0.358 gram of nitrogen. The solution for arginin contained 0.657 gram of nitrogen, and after the same treatment for separating the py-

rimidin bases as described above, it contained 0.223 gram of nitrogen. These results are summarized in Table V.

TABLE V.

DISTRIBUTION OF NITROGEN IN EXTRACT WITH 5 PER CENT. ACID.

	Grams and Percentage of Germ	Percentage of Total N of Germ	Percentage of N of Extract
Total N	10.65		
N in extract	9.58	89.99	100.00
Humin N	1.48	13.90	15.81
N removed by precipitation of			
(a) Xanthin bases	0.464	4.36	4.84
(b) Histidin and arginin	1.726	16.21	18.01
N in solution for histidin	0.454	4.27	4.74
“ “ “ “ “ (after precipitation			
and resolution)	0.358	3.36	3.73
N in solution for arginin	0.657	6.17	6.86
“ “ “ “ “ (after precipitation			
and resolution)	0.223	2.09	2.33
N in solution for lysin	2.83	26.55	29.50
N in solution for monamido compounds	1.704	16.00	17.78

Comparing Tables IV and V, the extract with 5 per cent. acid appears to contain a larger proportion of diamido compounds and a less proportion of monamido compounds, but the results were disappointing. The persistence of certain proteid reactions, the sticky, hygroscopic residues resisting all devices for crystallization, all point toward the presence of albumose, peptones, or similar bodies. The various reagents also failed to give as sharp separations as they should if the hydrolysis were complete: hence the use of the weaker acid was discontinued, although it is possible that with longer boiling it would have sufficed. Digestion with concentrated hydrochloric acid was also tried with no better results. Accordingly, for further work the stronger sulfuric acid was used.

The cel substance was stirred with nine times its weight of 33.33 per cent. sulfuric acid, allowed to stand over night, then heated in an evaporating dish on the water-bath. When all danger of frothing was over, the mixture was transferred to a flask fitted with a reflux condenser and boiled on a sand-bath for eight hours one day and six the next. After cooling and filtering, some water was added to the filtrate, and it was neutralized by the addition of the calculated amount of barium hydrate.

When the barium sulfate had completely settled, the supernatant liquid was siphoned off, the precipitate stirred up with boiling water, heated to boiling, settled over night, and again siphoned. This was repeated until the wash water was nearly colorless. The extract and wash water were united, acidified with acetic acid (if there is large excess of barium present, it is well to remove it by carbon dioxid), concentrated on the water-bath, cooled, and filtered to remove any leucin and tyrosin that may crystallize out. The filtrate was diluted to about one and a half liters for each 100 grams of cell substance, made decidedly acid with nitric acid, and 20 per cent. silver nitrate solution added as long as it gave a precipitate. It was left over night to settle, and the silver precipitate of xanthin bases filtered out. To this filtrate excess of silver nitrate and barium hydrate were added to remove arginin and histidin. After their removal, silver and barium were precipitated by hydrochloric and sulfuric acids, these inorganic precipitates boiled out with water several times, the filtrate and wash water united and concentrated. The solution, which should contain some 5 per cent. of acid, was treated with a 50 per cent. solution of phosphotungstic acid as long as it gave an immediate precipitate. The precipitate was rubbed up with 5 per cent. sulfuric acid, carefully washed with the same solution, and filtered with suction. The heavy white precipitate was again rubbed up with water, hot saturated solution of barium hydrate added until the mixture was no longer acid, settled over night, and the supernatant liquid siphoned off. The precipitate consisting of barium phosphate, tungstate, etc., was washed several times with hot barium hydrate solution, decanted, and finally filtered by suction. The filtrate and wash water were united, and barium was removed as carefully as possible, first by running in carbon dioxid, and then by adding ammonium carbonate to the solution. This precipitate, like all the other inorganic ones, was boiled out several times with water, and the washings added to the original filtrate. The resulting liquid was concentrated nearly to dryness on the water-bath, the residue taken up with water, filtered to remove barium carbonate, and again concentrated to a thick syrup.

This alkaline syrup was vigorously stirred with alcohol, and

then with an alcoholic solution of picric acid. Sometimes a crystalline precipitate came down at once, sometimes there was a viscous mass like molasses candy, which became granular or crystalline after long kneading and stirring as Fischer and Weigert suggest. When picric acid would no longer give a precipitate even on standing, the crystals were filtered out by suction, washed with alcohol, and dried on a porous plate. On concentration the alcoholic mother liquor became gummy and viscous, but no more crystals were obtained. The crude picrate was recrystallized from hot water several times. On dissolving there was much sediment which mainly filtered out, but on concentration more appeared upon the sides of the vessel. The loss of substance by the first recrystallization was very large; as it became pure, however, it crystallized like an inorganic salt. All mother liquors were treated with hydrochloric acid to remove picric acid, reprecipitated with phosphotungstic acid, the precipitate worked up as before, and a further crop of crystals obtained. The crystals are slender, yellow, silky, felted needles or prisms. On heating in a melting-point tube, the substance begins to change color at 216°C ., and is very dark at 230°C . Heated side by side with lysin picrate from fibrin and from gelatin, they agree within a degree. The authorities all agree that lysin picrate turns black at 230° – 232°C ., while Kutscher and Lohmann also say that it begins to change color at 215°C .

To change the picrate into chlorid, 2 grams were dissolved in 30 c.c. of hot water, 5 c.c. of concentrated hydrochloric acid added, cooled, the picric acid filtered out, and washed with water containing hydrochloric acid. The filtrates were shaken out with ether as long as there was any yellow color. The solution should be colorless or nearly so; if it is not, it can be decolorized by treatment with animal charcoal. The solution was evaporated nearly to dryness, first on the water-bath, and finally in a desiccator. When down to a thick syrup, stirring gave crystals. These were recrystallized out of hot water containing hydrochloric acid, giving long colorless prisms, which melt at 192°C ., again agreeing with the corresponding salt from gelatin and fibrin. Henze says that lysin chlorid becomes soft at 193°C ., and melts at 195°C .; Lawrow says that it has no sharp melting point, but begins to melt at 194° – 195°C .

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Henderson collected samples melting from 190° to 200° C. prepared by different individuals from widely different sources. By careful purification he obtained from each sample a product melting at 192° – 193° C. Thus it would appear that the apparent discrepancy in melting point is due to impurities. Reactions, crystalline form, properties, and melting (or decomposing) point show that the picrate and chlorid from the germ are identical with lysin picrate and chlorid from gelatin and from fibrin. Thus the presence of one of the hexon bases in the bacterial cell has been established, and another point of resemblance between bacterial and other proteid has been demonstrated.

SUMMARY.

Elementary analyses show that age, conditions of growth, and especially the composition of the nutrient medium cause bacteria of the same strain to differ widely in elementary composition. Proteid, nucleoproteid, nucleic acid, protamin, fat, wax, lecithin, glycogen, and other carbohydrates have all been reported as obtained from the bacterial cell in varying degrees of purity. Cellulose seems to be present in certain species, but by no means in all. Besides the preparations mentioned above, crystalline compounds have been prepared and purified, proving the presence in the cell of xanthin bases, pentose, fatty acids, and perhaps thymine and uracil. Toxins, enzymes, and agglutinins have been split off from the cell; but more progress has been made in determining their physiological action than their chemical nature.

The cell substance of *Bacillus coli communis*, as prepared for this study, yielded about 8.5 per cent. of ash, and 3 per cent. of phosphorus. Although it had been hardened by contact with alcohol, it is completely dissolved by successive treatment with dilute acid and dilute alkali. Heating with 1 per cent. sulfuric acid causes cleavage apparently along definite lines. The extract contains carbohydrate, much phosphorized nitrogenous matter and bases, but neither proteid, protamin, nor nucleoproteid. The decomposition products suggest that the cell is largely made up of nuclein or glyconucleoproteid; but even this dilute acid suffices to break off the carbohydrates, and to split up the proteid. Alcohol precipitates from this extract a hygroscopic,

toxic body, readily acted upon by the air when moist, apparently made up of sulfate, nucleic acid, and colon toxin. The alkaline extract contains carbohydrate and a nucleo-compound. No evidence of cellulose was found.

By digestion with stronger acid, xanthin and hexon bases were obtained. Lysin was isolated as picrate, purified, and transformed into the chlorid, and proved to be identical with lysin picrate and chlorid from other sources.

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